Biol Blood Marrow Transplant 19 (2013) 692–702

Biology

Dynamic Change and Impact of Myeloid-Derived Suppressor Cells in Allogeneic Bone Marrow Transplantation in Mice

Dapeng Wang [1,2], Yu Yu [1,2], Kelley Haarberg [1,2], Jianing Fu [1,2], Kane Kaosaard [1,2], Srinivas Nagaraj [3,4], Claudio Anasetti [1,2,3,5], Dmitry Gabrilovich [1,5,6], Xue-Zhong Yu [1,2,3,5,*]

[1] *Department of Immunology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida*
[2] *Department of Blood and Marrow Transplantation, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida*
[3] *Department of Pathology and Cell Biology, College of Medicine, University of South Florida, Tampa, Florida*
[4] *Department of Internal Medicine, College of Medicine, University of South Florida, Tampa, Florida*
[5] *Department of Oncologic Sciences, College of Medicine, University of South Florida, Tampa, Florida*
[6] *Department of Molecular Medicine, College of Medicine, University of South Florida, Tampa, Florida*

Article history:
Received 9 November 2012
Accepted 15 January 2013

Key Words:
MDSC
BMT
GVHD
Tumor relapse

A B S T R A C T

Myeloid-derived suppressor cells (MDSCs) are a group of myeloid cells composed of hematopoietic progenitor cells, immature macrophages, dendritic cells, and granulocytes, which accumulate in inflammatory diseases and various cancers. Here, we investigated the dynamic changes and effects of MDSCs in graft-versus-host disease (GVHD) development and/or tumor relapse after syngeneic and allogeneic bone marrow transplantation (BMT). We found that adding functional MDSCs in donor graft alleviated GVHD, whereas removal of MDSCs in vivo exacerbated GVHD. After T cell-deplete BMT, MDSCs transiently accumulated in the blood and spleen of recipients without GVHD. In contrast, after T cell-replete BMT, the levels of blood MDSCs were constantly elevated in recipients with GVHD. MDSC accumulation positively correlated with the severity of GVHD. Additionally, MDSC accumulation was further increased upon tumor relapse. Although MDSCs isolated from both syngeneic and allogeneic BMT recipients inhibited T cell proliferation in response to alloantigen stimulation ex vivo, MDSCs from the recipients with GVHD showed much higher suppressive potency compared with those from recipients without GVHD. These results indicate that MDSCs can regulate the immune response in acute GVHD, and possibly tumor relapse, subsequent to allogeneic BMT.

INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous cell population that includes immature myeloid cells and the progenitor cells of macrophages, dendritic cells (DCs), monocytes, and neutrophils. Accumulation of MDSCs was originally found in cancer patients [1-3]. Because MDSCs inhibit T cell activation and help tumor cells evade attack from the host immune system, they are regarded as "bad" cells with no beneficial effects to humans. Contrary to that opinion, findings indicate that MDSCs may play an immune regulatory role in infectious diseases, autoimmune diseases, and inflammatory diseases. Acute *Trypanosoma cruzi* infection has been found to induce T cell activation, increase the production of interferon-γ (IFN-γ), and subsequently lead to MDSC expansion [4,5]. In a mouse model of multiple sclerosis, accumulation of MDSCs was observed in the spleen and blood, and these cells were found to enter the central nervous system during the inflammatory phase of the disease [6]. The apparent function of accumulated MDSCs in infectious and inflammatory disease is to regulate the immune response in a "reasonable" scope and prevent tissue damage.

"Natural suppressor cells" were found in the spleens of clinical bone marrow transplantation (BMT) recipients and suppressed T cell proliferation under the stimulation of alloantigen or mitogen [7-11]. Their myeloid origin, accumulation, and suppressive function in individuals with cancer was confirmed 10 years later when excessive numbers of $CD34^+$ myeloid cells were noted in the blood of patients with head and neck squamous cell carcinoma [12,13]. Graft-versus-host disease (GVHD) is the result of attack of allogeneic donor T cells on multiple host organs and is a significant cause of morbidity and mortality after allogeneic BMT. Host myeloid-derived cells, such as DCs acting as antigen-presenting cells (APCs), are important mediators for the initiation of GVHD [14,15]. Donor APCs transferred with the graft or differentiated from hematopoietic progenitor cells drive more extensive tissue injury by cross-presentation of host alloantigens [16]. As the precursors of DCs and macrophages, the role of MDSCs in the pathogenesis of GVHD is not clear.

There are few studies on MDSCs in the setting of allogeneic BMT. It was reported that MDSCs accumulated in blood peaked in number at week 3 and returned to the physiological level at week 12 in minor histocompatibility-mismatched BMT recipients, suggesting a regulatory role of MDSCs in GVHD [17]. In vitro or in vivo cytokine stimulation–generated MDSCs alleviated GVHD in murine allogeneic BMT models [18,19]. It is unclear, however, how MDSC accumulation affects GVHD. In the current study, we

Financial disclosure: See Acknowledgments on page 701.

* Correspondence and reprint requests: Xue-Zhong Yu, MD, MS, H. Lee Moffitt Cancer Center & Research Institute, SRB-2, 12902 Magnolia Drive, Tampa, FL 33612-9497.
E-mail address: Xue.Yu@moffitt.org (X.-Z. Yu).

1083-8791/$ – see front matter © 2013 American Society for Blood and Marrow Transplantation.
http://dx.doi.org/10.1016/j.bbmt.2013.01.008

showed that differentiation of MDSCs with all-*trans* retinoic acid (ATRA) significantly aggravated GVHD, whereas supplement of MDSCs to donor graft alleviated GVHD, indicating MDSCs regulated GVHD development in vivo.

We further studied the kinetics of MDSC accumulation in allogeneic BMT recipients and tested their immune suppressive function in vitro. We found that MDSCs accumulated in the blood and spleens of BMT recipient mice (both allogeneic and syngeneic BMT) and gradually reduced to physiological levels if GVHD did not occur. MDSC accumulation in the blood was closely associated with the development of GVHD. Tumor relapse further increased MDSC accumulation in blood and spleen even in the recipients after allogeneic BMT. The accumulated MDSCs significantly inhibited allogeneic T cell proliferation.

METHODS

Mice

BALB/c (H-2^d), FVB/NCr (H-2^q, termed FVB), C57BL/6 (H-2^b, termed B6), and B6 × DBA2 F1 (H-$2^{b/d}$, termed B6D2F1) mice were purchased from the National Institutes of Health (Bethesda, MD). Luciferase transgenic mice on B6 background were kindly provided by Dr. Robert Negrin (Stanford University, Stanford, CA). All the mice were housed in pathogen-free conditions at H. Lee Moffitt Cancer Center. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

Reagents and Chemicals

The following antibodies specific for mouse antigens were used for cell surface staining for flow cytometry or T cell purification: anti-Gr-1 FITC (clone RB6-8C5), anti-Gr-1 biotin (clone RB6-8C5), anti-CD4-FITC, anti-CD4-APC (L3T4), anti-CD8α-FITC, anti-CD8α-APC, anti-H-$2K^b$-FITC, anti-CD11b-PE-Cy7, anti-CD11b-biotin, anti-B220-biotin, anti-DX5-biotin, and anti-Ter119-biotin were purchased from eBioscience (San Diego, CA). Anti-Ly6G FITC (clone 1A8) and anti-Ly6C APC (clone AL-21) were purchased from BD Bioscience (San Jose, CA). Anti-Thy1.2 (clone 30H12), anti-CD3 (clone 2C11), and anti-CD28 (clone 37.51) were purchased from BioXCell (West Lebanon, NH). Young rabbit complement was purchased from GTI Diagnostics (Waukesha, WI). ATRA was purchased from Sigma Aldrich (St. Louis, MO). ATRA was dissolved in dimethyl sulfoxide (DMSO), stored in −20° freezer, and diluted in phosphate-buffered saline (PBS) before use. Carboxyfluorescein diacetate, succinimidyl ester (CFSE) was purchased from Invitrogen (Grand Island, NY). Catalase and uric acid were purchased from Sigma Aldrich L-NG-Monomethylarginine, Acetate Salt (L-NMMA). and n-omega- Hydroxy-nor-L-arginine (Nor-NOHA) were purchased from Cayman Chemical (Ann Arbor, MI).

Cell Preparation

T cells were purified through negative selection using magnetic bead depletion of non–T cells. Briefly, after lysis of red blood cells, spleen and lymph node cells were incubated with biotin-conjugated anti-CD11b, anti-B220, anti-DX5, and anti-Ter119 for 15 minutes in .1% bovine serum albumin (BSA)-PBS at 4°C. Cells were subsequently washed and incubated with anti-biotin beads (Miltenyi, Auburn, CA) for 15 minutes at 4°C in .1% BSA-PBS, and antibody-bound cells were removed magnetically using LS column (Miltenyi). T cell purity was normally over 95%. Mouse bone marrow cells were harvested by flushing femurs and tibias with 1% fetal calf serum RPMI-1640. T cells were removed by incubation with anti-Thy-1 antibody (4°C, 30 min) followed by incubation with young rabbit complement (37°C, 45 min). MDSCs were isolated from recipient spleens and bone marrow by incubation with anti-Gr-1 biotin antibody (4°C, 15 min) followed by washing and incubation with anti-biotin beads (4°C, 15 min). Cells other than MDSCs were removed magnetically by LS column sorting. MDSC purity was typically between 90% and 95%.

Bone Marrow Transplantation

BALB/c mice were exposed to total body irradiation (TBI) at 800 cGy (a single dose) using a Shepherd Mark I Cesium Irradiator (J.L. Shepherd and Associates, San Fernando, CA) at day −1. If B6 or B6D2F1 mice were used as recipients, the dose of TBI was 1,200 to 1,300 cGy (two split doses). Irradiated recipients received a single intravenous injection in the lateral tail vein of T cell–depleted bone marrow cells (TCD-BM) with or without T cells within 24 hours. The severity of GVHD was assessed by mice survival and loss of body weight. In addition, systemic GVHD was scored based on 5 clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity. Individual mice were scored 0 to 2 for each criteria and 0 to 10 overall [20].

Leukemia or Lymphoma Models

To mimic clinical tumor relapse, we used lymphoma or leukemia cell lines in the B6 → BALB/c and B6 →B6D2F1 BMT models, respectively. In BALB/c recipients, the A20 B lymphoma cell line transduced with a luc/neo plasmid (A20-luc) was used to allow for visualization of tumor dissemination. Mice received 800 cGy TBI on day −1. On day 0, BALB/c recipients received grafts containing 5×10^6 TCD-BM with or without $.1 \times 10^6$ T cells and various numbers of A20-luciferase-expressing cells (A20-Luc) as indicated in corresponding figures. In the B6→B6D2F1 BMT model, mice received 1,200 to 1,300 cGy TBI on day −1. On day 0, B6D2F1 recipients received grafts containing 5×10^6 TCD-BM with 2 to 4×10^6 T cells and 1×10^4 host type P815 (DBA/2-derived, H-2^d) tumor cells.

To further mimic clinical tumor relapse, we also used an inducible model of chronic myeloid leukemia (CML). MSCV2.2 expressing the human p210 bcr/abl cDNA and enhanced green fluorescent protein (EGFP) driven by an internal ribosome entry site (Mp210/GFP) was a gift from Dr. Warren Shlomchik (Yale, New Haven, CT). Retroviral supernatants were generated by way of transient transfection of the Platinum-E Retroviral Pack cell line (Cell Biolabs, San Diego, CA) as described previously [16,21]. In short, on day −1, 4×10^6 Platinum-E Retroviral Pack cells were seeded on 6-cm plates in Dulbecco-modified Eagle medium with 10% fetal calf serum. On day 0, the cells were transfected with 8 μg Mp210/GFP using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Thirty-six hours after transfection, the media was changed. Retroviral supernatants were harvested 12 hours later, filtered through .45-μm screens, and stored in aliquots at −80°C.

To establish CML in mice, BALB/c were injected with 5 mg of 5-fluorouracil (Sigma-Aldrich) on day −6. On day −2, BM cells were harvested and cultured in prestimulation medium (Dulbecco-modified Eagle medium, 15% fetal bovine serum (FBS), 6 ng/mL interleukin [IL]-3, 10 ng/mL IL-6, and 10 ng/mL Stem cell factor (SCF) all cytokines were from Peprotech, Rocky Hill, NJ). On day −1 and 0, cells were resuspended at 2×10^6/mL in prestimulation medium with the addition of the retroviral supernatants Polybrene (4 μg/mL; Sigma-Aldrich) and HEPES (100 mM) and underwent spin infection (1,000 ×g, 37°C, for 1.5 hours). BM cells were returned to a CO_2 incubator for 2 hours after each spin infection. On day 0, 2×10^5 infected BM cells along with 5×10^6 TCD-BALB/c BM cells were transferred to lethally irradiated BALB/c mice. BALB/c recipients were bled weekly from week 1, and $CD11b^+GFP^+$ leukemia cells were monitored by flow cytometry. Premorbid CML mice were killed, and 2×10^6 CML BM and splenocytes along with 5×10^6 TCD-B6 BM cells were transferred to lethally irradiated BALB/c mice to establish spontaneous CML in recipient mice.

In Vivo Bioluminescence Imaging

Mice were given an intraperitoneal injection of luciferin (150 mg/kg body weight) and then anesthetized with isoflurane gas using an XGI Gas anesthesia system (Xenogen, Cranbury, NJ) and imaged using the IVIS imaging system (Xenogen, Cranbury, NJ) to assess bioluminescence (BLI) 10 minutes after injection of the substrate. Imaging data were analyzed and quantified with Living Image Software (Xenogen) as described in our previous work [22].

Mixed Lymphocyte Reaction

B6 T cells and BALB/c splenic APCs were isolated from spleen and lymph node using magnetic column selection with biotin antibodies and anti-biotin magnetic beads. TCD-splenic cells from BALB/c mice were irradiated at 2,000 cGy. CFSE-labeled 2×10^5 purified B6 T cells were used as responder and 6×10^5 BALB/c TCD-splenic cells as stimulators. Cells were cultured in 96 round-bottom plates for 5 days, and the proliferation of B6 T cells was measured by flow cytometry.

T Cell Proliferation

A 48-well plate was coated with 10 μg/mL anti-CD3 antibody and 5 μg/mL anti-CD28 antibody in PBS at 4°C, overnight. The coated plate was washed with 1% fetal bovine serum RMPI-1640 two times before use. CFSE-labeled 2×10^5 purified B6 T cells were seeded in the plate with different concentrations of ATRA. T cell proliferation was measured by CFSE dilution in 72 hours.

Statistical Analysis

The log-rank test was used to detect statistical differences in recipients' survival in GVHD experiments. Student's *t*-test was used in other experiments.

RESULTS

MDSCs Accumulate in Allogeneic BMT Recipients

To relate MDSCs to GVHD development, we monitored the presence of MDSCs over time in the blood and spleens of

recipients of syngeneic or allogeneic BMT. In B6 → B6 syngeneic BMT recipients, MDSCs accumulated in blood and spleens at week 2 but declined gradually to the levels of the control mice without BMT. No significant difference was detected between recipients that underwent transplantation with TCD-BM alone and TCD-BM plus T cells at any time point tested (Figure 1A,C). In the B6 → BALB/c allogeneic BMT model, MDSC accumulation was also transient in recipients of TCD-BM alone; however, MDSC accumulation was prolonged in recipients of TCD-BM plus T cells (Figure 1B,C). Because severe hypocellularity was caused by acute GVHD in the recipients of TCD-BM plus T cells, the absolute numbers of MDSCs in recipient spleen at week 4 were comparable between two groups (data not shown).

Anti-Gr-1 antibody binds to the common epitope of Ly6G and Ly6C. Accordingly, MDSCs can be further divided into granulocytic MDSCs ($CD11b^+Ly6G^+Ly6C^{low}$) and monocytic MDSCs ($CD11b^+Ly6G^-Ly6C^{hi}$), as shown in Figure 1D. Previous studies showed these two MDSC populations had different functions in cancer and infectious disease [6,23,24]. We studied the accumulation of both MDSC populations in syngeneic and allogeneic BMT. Similar to the $CD11b^+Gr\text{-}1^+$ population, granulocytic and monocytic MDSC subpopulations accumulated at week 2 and quickly declined in the blood and spleens of recipients without GVHD (Figure 1E,F). In contrast, accumulation of both subpopulations maintained at high levels for a long period of time in the recipients with GVHD. To exclude the possibility that this phenomenon was BMT model specific, we performed similar experiments using another major histocompatibility complex (MHC)-mismatched BMT model (FVB/N → BALB/c) and also found that the sustained MDSC accumulation was only observed in BM-plus-splenocyte recipients with GVHD (Supplementary Figure S1).

Accumulated MDSCs Are Immune Suppressive

To test the function of $CD11b^+Gr\text{-}1^+$ cells accumulated in BMT recipients, we performed a series of mixed lymphocyte reaction (MLR) using CFSE-labeled T cells from B6 mice as responders and TCD-splenocytes from BALB/c mice as stimulators. As shown in Figure 2A, MDSCs isolated from allogeneic recipients at week 2 significantly inhibited T cell proliferation. Similarly, MDSCs from syngeneic recipients 2 weeks after BMT were also immune suppressive (Figure 2B).

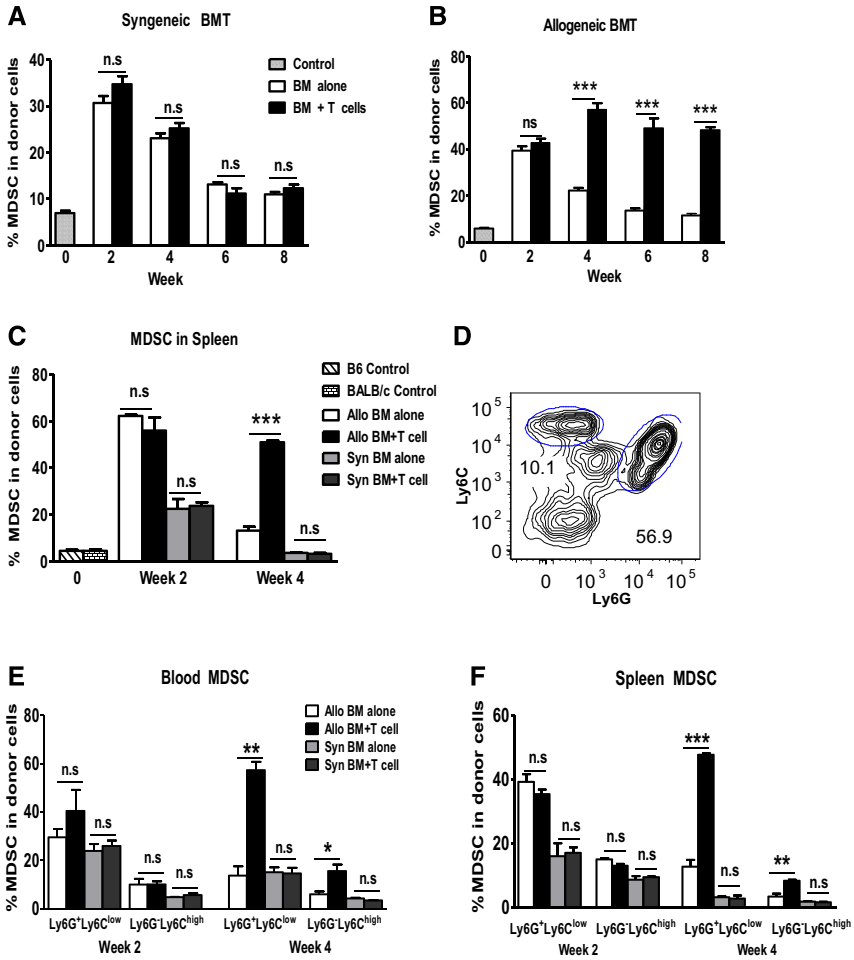

Figure 1. MDSCs accumulated in recipients after BMT. BALB/c or B6 mice (n = 4) were lethally irradiated and underwent transplantation with 5×10^6 TCD-BM with or without 1×10^6 T cells from B6 donors. The percentage of donor-derived MDSCs, $H\text{-}2K^{b+}CD11b^+Gr\text{-}1^+$ cells, in blood and spleen was measured with flow cytometry every other week after BMT. (A) Kinetics of blood MDSCs in syngeneic BMT recipients from week 2 to week 8. (B) Kinetics of blood MDSCs in allogeneic BMT recipients from week 2 to week 8. (C) Kinetics of splenic MDSCs in both allogeneic and syngeneic BMT recipients at week 2 and week 4. (D) Splenocytes were stained for expression of $H\text{-}2K^b$, CD11b, Ly6G, and Ly6C. The expression of Ly6G and Ly6C is shown on $H\text{-}2K^{b+}CD11b^+$ cells, and MDSCs were defined as $Ly6G^+Ly6C^{low}$ and $Ly6G^-Ly6C^{high}$ subpopulations. (E) Data shown are two MDSC subpopulations in the blood of allogeneic and syngeneic BMT recipients at week 2 and week 4. (F) Data shown are two MDSC subpopulations in the spleen of allogeneic and syngeneic BMT recipients at week 2 and week 4. Similar results were obtained from three independent experiments, and one representative experiment was shown. n.s. indicates no significant difference, * $P < .05$, ** $P < .01$, *** $P < .001$.

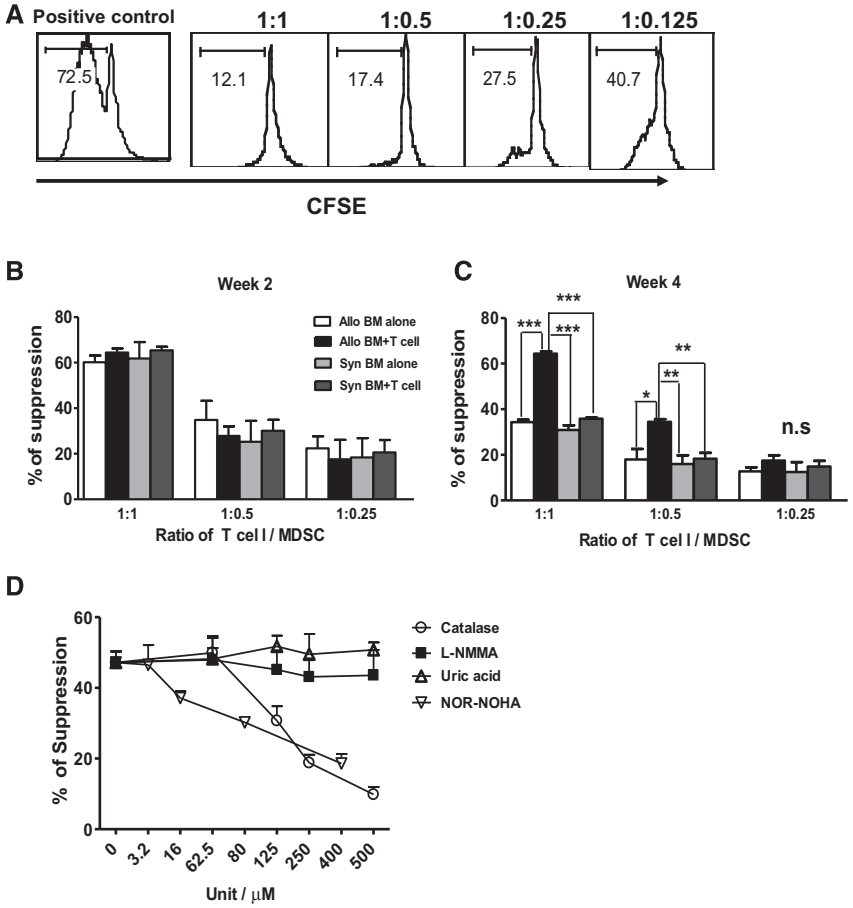

Figure 2. MDSCs accumulated in BMT recipients were immune suppressive. BALB/c or B6 mice (n = 4) were lethally irradiated and underwent transplantation with 5×10^6 TCD-BM with or without 1×10^6 T cells from B6 donors. Recipient mice were killed on day 14 and day 28, and splenic MDSCs were isolated for testing their suppressive function in vitro. CFSE-labeled B6 T cells were used as responders, and TCD-irradiated splenocytes from BALB/c mice were used as stimulators at the ratios of T cell/MDSC indicated. (A) Proliferation of CFSE-labeled B6 $CD8^+$ T cells is inhibited in the presence of MDSCs from the BALB/c recipients of TCD-BM + T cells. (B) Suppressive activity of MDSCs from allogeneic and syngeneic recipients 2 weeks after BMT. % of suppression = (% CSFE-diluted cells without MDSCs − % CSFE-diluted cells with MDSCs) / (% CSFE-diluted cells without MDSCs − % CSFE-diluted cells without stimulators) × 100%. (C) Suppressive activity of MDSCs from allogeneic and syngeneic recipients 4 weeks after BMT. (D) The effects of catalase, L-NMMA, uric acid, or Nor-NOHA on the suppressive function of MDSCs from allogeneic BM plus T cell recipients. Drug concentration is showed in μM except for catalase in units/mL. The ratio of T cell to MDSC is 1:.5. One representative experiment from 3 independent experiments was shown. n.s. indicates no significant difference, * $P < .05$, ** $P < .01$, *** $P < .001$.

Interestingly, MDSCs isolated from allogeneic recipients with GVHD 4 weeks after BMT showed significantly higher suppressive activity than those from recipients without GVHD (Figure 2C). Because arginase 1, inducible nitric oxide synthase, reactive oxygen species, and peroxynitrite are potential mediators for immune suppression by MDSCs [25-28], we tested which molecules mediated MDSCs' immune suppression by using specific inhibitors to block each of these pathways. As shown in Figure 2D, catalase (H_2O_2 scavenger, antagonize reactive oxygen species) and Nor-NOHA (arginase inhibitor) efficiently reversed the immune suppressive function of MDSCs, whereas L-NMMA (inducible nitric oxide synthase inhibitor) and uric acid (peroxynitrite scavenger) did not have an effect. Therefore, the suppressive function of MDSCs in the setting of BMT was arginase-1 and reactive oxygen species dependent. In addition, we explored the possibility that the suppressive function of MDSCs was MHC dependent. We observed similar suppression of the proliferation of MHC-matched and unmatched T cells, suggesting MDSCs can mediate immune suppression in an MHC-independent manner (data not shown).

Accumulation of MDSCs in BMT Recipients Correlated with the Severity of GVHD

To test the hypothesis that the accumulation of MDSCs correlated with the severity of GVHD, we transplanted various numbers of T cells from B6 donors into allogeneic BALB/c recipients to induce GVHD with various severities and compared the kinetics of MDSC accumulation across groups of recipients. According to weight loss (Figure 3A), the severity of GVHD positively correlated with the number of donor T cells transplanted. MDSC accumulation occurred in the peripheral blood of all recipients 2 weeks after BMT (Figure 3B). Recipients that underwent transplantation with $.5 \times 10^6$ donor T cells showed the highest percentage of MDSC accumulation among all groups at weeks 4 and 6. Recipients of $.1 \times 10^6$ T cells displayed moderate MDSC accumulation, whereas recipients of $.02 \times 10^6$ T cells showed no difference in MDSC accumulation compared with BM alone. Furthermore, donor T cells induced MDSC accumulation in the recipients' spleens in a dose-dependent manner at week 6 (Figure 3C). Thus, the accumulation of MDSCs positively correlated to the severity of GVHD after allogeneic BMT.

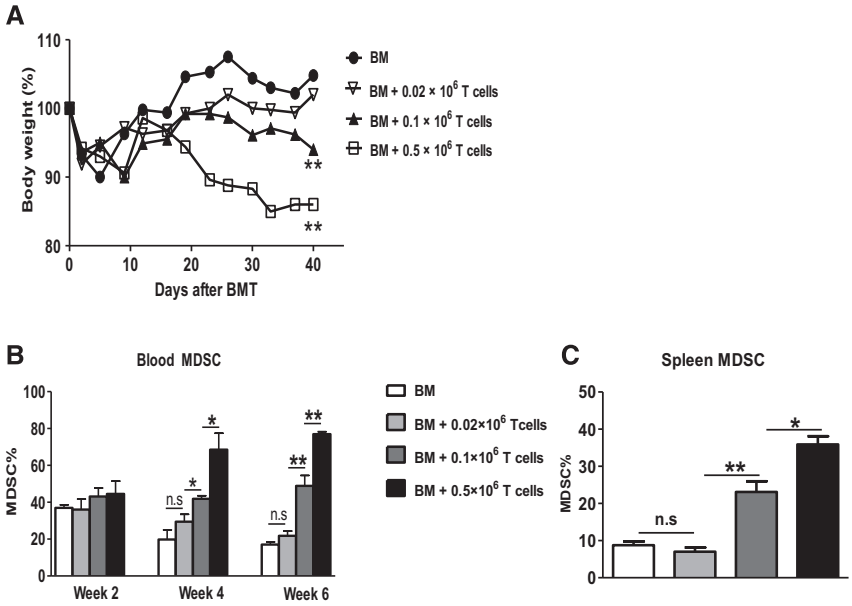

Figure 3. Accumulation of MDSCs in BMT recipients correlated with the severity of GVHD. BALB/c (n = 4) were lethally irradiated and underwent transplantation with 5×10^6 TCD-BM plus various numbers of T cells from B6 donors. The percentage of MDSCs in blood was measured by flow cytometry every other week after BMT. Splenic MDSCs were measured at the end of study (week 6). Recipient body weight was measured twice a week, and recipient body weight changes are shown (A). (B) Kinetics of blood MDSCs in BMT recipients. (C) Percentage of splenic MDSCs is depicted at the end of study. Similar results were obtained from 2 independent experiments, and one representative experiment was shown. n.s. indicates no significant difference, * $P < .05$, ** $P < .01$, *** $P < .001$.

Removal of MDSCs Aggravated GVHD

We next explored whether accumulated MDSCs were immune suppressive and regulated GVHD in vivo. ATRA induces apoptosis of granulocytic MDSCs and differentiates monocytic MDSCs to mature myeloid cells including macrophage and DCs [29-32]. ATRA treatment significantly reduced the absolute number of MDSCs in the spleens of recipients that underwent transplantation with TCD-BM alone (Figure 4A). Splenic reconstitution, survival, and weight loss were not affected by ATRA treatment (Figure 4B-D). To test the potential impact of MDSCs in the development of GVHD, we used ATRA to reduce MDSC numbers after allogeneic BMT. ATRA treatment significantly reduced the total number of MDSCs in recipient spleens (Figure 4E). Recipients treated with ATRA died from GVHD significantly sooner than vehicle-treated controls (Figure 4F). To exclude the possibility that ATRA accelerated GVHD by enhancing T cell activation, we tested the effect of ATRA on T cell response to anti-CD3/CD28 stimulation in vitro and found that T cell proliferation was not affected by treatment with ATRA in a wide range of concentrations (Supplementary Figure S2). Hence, our current data suggest that ATRA-induced reduction of MDSCs likely contributed to the accelerated GVHD.

Adoptive Transfer of In Vivo-Generated MDSCs Alleviated GVHD

Because removal of MDSCs was associated with accelerated GVHD, we further tested the hypothesis that transferring MDSCs at the time of BMT would alleviate GVHD. Granulocyte colony-stimulating factor (G-CSF) was used to induce accumulation of functional MDSCs in mice, as previously shown [33]. MDSCs were defined as $CD11b^+Gr\text{-}1^+$ cells, including the Gr-1 high and Gr-1 medium cell populations (Figure 5A). Percentages of MDSCs were much higher in G-CSF–treated B6 mice, as shown in Figure 5B. G-CSF–primed MDSCs inhibited T cell proliferation under allogeneic stimulation in vitro (data not shown). Cotransferring splenic MDSCs from G-CSF–treated B6 mice significantly alleviated GVHD in BALB/c recipients that underwent transplantation with TCD-BM plus T cells from normal B6 donors ($P < .05$). MDSCs isolated from the BM of G-CSF–treated mice delayed GVHD, but $Gr\text{-}1^+CD11b^+$ cells isolated from the BM of vehicle-treated mice accelerated GVHD as reflected by survival, body weight loss, and clinical scores (Figure 5D-F). Consistent with clinical manifestations, total cells and donor-derived B cells in recipient spleens were also significantly preserved by additional MDSCs (Figure 5G,H). Additional MDSCs had no effect on donor engraftment, as full-donor chimerism was achieved in all the recipients (Supplementary Figure S3). We did not test MDSCs from the spleens of PBS-treated mice due to low numbers of MDSCs in these mice.

To measure the effect MDSCs on donor T cell expansion, we repeated the BMT experiment by using T cells from luciferase transgenic mice on the B6 background. Consistent with recipient lethality and weight loss, transferred splenic MDSCs from G-CSF–treated B6 mice also significantly reduced T cell expansion, as reflected by strength of the BLI signal. Conversely, BM-derived MDSCs from vehicle-treated mice enhanced T cell expansion (Figure 5C). In addition, vehicle-treated BM $Gr\text{-}1^+CD11b^+$ cells significantly increased IFN-γ production by donor $CD4^+$ and $CD8^+$ T cells, whereas G-CSF-treated splenic MDSCs significantly decreased IFN-γ production by $CD8^+$ T cells in the liver (Figure 5I) and in the spleen (Figure 5J). Hence, cotransfer of functional MDSCs at the time of allogeneic BMT significantly reduced T cell activation and expansion and thus alleviated GVHD.

Tumor Relapse Further Increased MDSCs Accumulation

Originally, MDSC accumulation was found in cancer patients, and tumor relapse is a major threat for BMT patients. To study the affects of tumor relapse on MDSC accumulation after BMT, we quantified MDSC accumulation

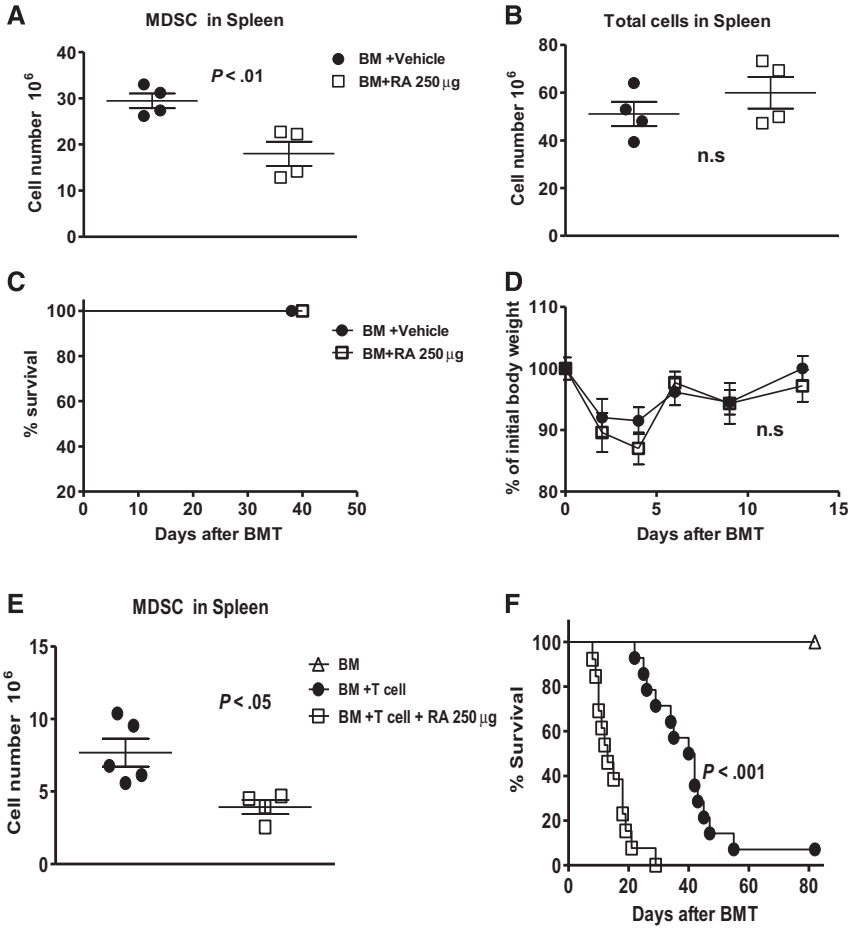

Figure 4. Removal of MDSC using ATRA aggravated GVHD. BALB/c mice were lethally irradiated and underwent transplantation with 5×10^6 TCD-BM alone (A-D) or with 1×10^6 T cells (E and F) from B6 donors. Recipient mice were treated with ATRA (RA, 1.25 mg/kg body weight) or solvent control via i.p. injection every other day from day 0 to day 12. Some mice were euthanized on day 14 to analyze the accumulation of MDSCs in the spleen. (A) ATRA treatment reduced MDSC number in the spleen of the recipients with TCD-BM alone. (B) ATRA treatment did not affect spleen reconstitution without GVHD. ATRA treatment had no effect on survival (C) or body weight (D) in the recipients of with TCD-BM alone. In the recipients that underwent transplantation with TCD-BM plus T cells, ATRA treatment reduced MDSC number in the spleen (E), aggravated GVHD, and accelerated lethality (F) (n = 13-14). (A-E) show representative data from three replicate experiments that produced similar results. (F) shows pooled results from 3 replicate experiments. n.s. indicates no significant difference, * $P < .05$, ** $P < .01$, *** $P < .001$.

in the presence or absence of tumor relapse. Lethally irradiated BALB/c mice underwent transplantation with TCD-BM and T cells from B6 donors with or without different numbers of A20-Luc lymphoma cells. Recipients that underwent transplantation with TCD-BM alone and 1×10^4 A20-Luc cells quickly relapsed, but an additional $.1 \times 10^6$ donor T cells essentially prevented lymphoma relapse (Figure 6A, B). Even at a high dose of lymphoma (5×10^4 A20-Luc cells), additional donor T cells were able to reduce the rate of tumor growth (Figure 6A, B). By examining the presence of MDSCs in recipient blood and spleens, we found that tumor relapse caused significantly higher levels of MDSC accumulation in the recipients of TCD-BM alone without GVHD and in the recipients of TCD-BM plus T cells with GVHD (Figure 6C, D). There was no difference in MDSC accumulation among the recipients of TCD-BM plus T cells with or without tumor infusion, however, as long as relapse was prevented. To exclude the possibility that this phenomenon was BMT model or tumor type specific, we performed similar experiments using another MHC-mismatched BMT model (B6 → B6D2F1) with p815-luciferase-expressing (P815-Luc) mastocytoma cells. Under these experimental conditions, only mild and self-limited GVHD was observed, and tumor relapse further increased MDSC accumulation in recipients with or without GVHD (Supplementary Figure S4). Thus, we conclude that tumor relapse enhanced MDSC accumulation after BMT regardless of GVHD.

Because transplantable tumors may not represent well clinical leukemia/lymphoma relapse, we further established a "spontaneous" CML mouse model by transfecting BM with P210 bcr/abl, which was then transferred into lethally irradiated syngeneic hosts as described in the Methods section. CML underwent transplantation with TCD-BM alone or plus T cells from B6 donors. CML growth was monitored by measuring GFP^+CD11b^+ cells and MDSCs as $Gr\text{-}1^+CD11b^+$ cells in recipient blood and spleens. Within 3 weeks, CML relapse was evident in BALB/c recipients that underwent transplantation with BM and splenocytes from CML mice, as GFP^+CD11b^+ cells were present in the blood and spleens of these recipients but not control recipients (Figure 7A). Accordingly, the percentages of MDSCs in blood and spleens were significantly higher in CML mice compared with controls without CML (Figure 7A). In the presence of donor T cells, the growth of CML was dramatically reduced (Figure 7B,C). Consequently, the increased MDSC accumulation by CML was reverted.

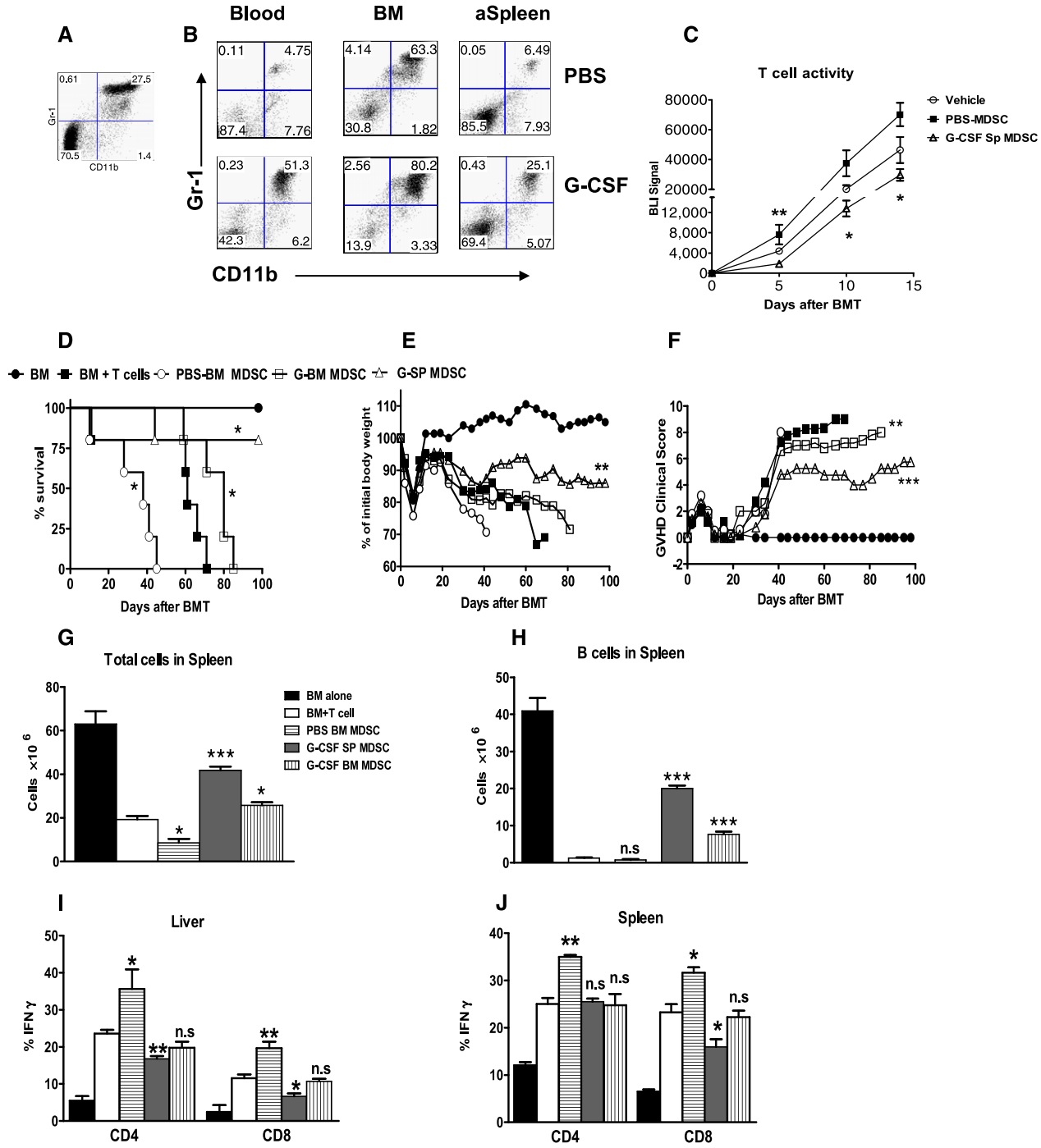

Figure 5. Cotransferring functional MDSCs alleviated GVHD after allogeneic BMT. B6 mice were subcutaneously injected 10 μg per mouse with human G-CSF or PBS daily from day 0 to day 4. Four hours after the last injection, MDSCs were isolated from the spleen and BM. MDSCs were cotransferred at 2×10^6 per mouse to lethally irradiated BALB/c mice that received TCD-BM and 1×10^6 T cells from B6 donors. Body weight was monitored twice a week, and survival was recorded daily. In some experiments, donor T cells were isolated from luciferase transgenic B6 mice to monitor the in vivo proliferation. (A) Splenocytes were stained for the expression of H-2K^b, CD11b, and Gr-1, and MDSCs were defined as H2K^{b+}/CD11b$^+$/Gr-1$^+$. (B) MDSCs accumulated in G-CSF–treated mice spleen, bone marrow, and blood. (C) Donor T cell proliferation in vivo reflected by average BLI signal at days 5, 10, and 14 after BMT. Recipient survival (D), body weight changes (E), and GVHD clinical score (F) are shown. Donor engraftment is shown by total spleen cell number (G) and B cell number (H) at day 28. (I and J) show the percentages of IFN-γ–secreting T cells in recipient spleen or liver at day 14. The data represent one of 3 replicate experiments. *P compared with BM+T cell group. n.s. indicates no significant difference, * $P < .05$, ** $P < .01$, *** $P < .001$.

Composition of MDSCs was Similar in the Recipients with GVHD or Tumor Relapse

Both GVHD and tumor relapse after BMT enhanced MDSC accumulation. We further asked whether the composition of MDSCs was different under these 2 situations. B6→ BALB/c BMT plus A20 tumor model was used, and phenotypes of MDSCs were measured 3 weeks after BMT (Figure 8A). We found that both granulocytic (CD11b$^+$Ly6G$^+$Ly6C^{low}) and monocytic (CD11b$^+$Ly6G$^-$Ly6C^{hi}) MDSCs were significantly increased in GVHD or tumor relapse, although more

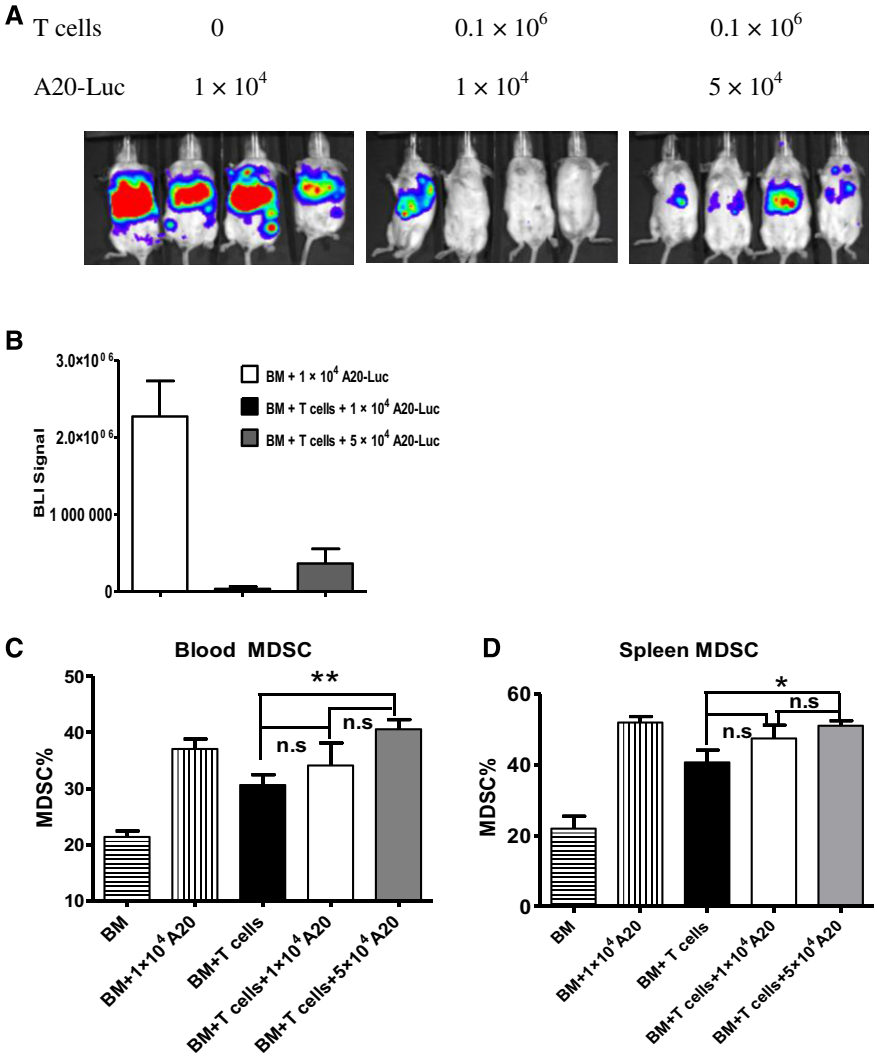

Figure 6. A20 tumor relapse further increased MDSC accumulation. BALB/c mice (n = 4) were lethally irradiated and underwent transplantation with TCD-BM plus .1 × 10^6 B6 T cells with various numbers of A20-Luc cells. The A20-Luc growth in vivo was monitored by BLI. The percentages of MDSCs in blood and spleen were determined with flow cytometry. (A) A20-Luc growth in vivo, reflected by BLI signal strength. (B) Average intensity of BLI signal for each experimental group. (C) The percentage of blood MDSCs at day 14. (D) The percentage of spleen MDSCs at day 18. Similar results were obtained from 2 independent experiments and 1 representative experiment was shown. n.s. indicates no significant difference, * $P < .05$, ** $P < .01$, *** $P < .001$.

dramatically in the former (Figure 8B). However, the ratios of granulocytic versus monocytic MDSC were comparable among 3 groups, indicating the composition of MDSCs was not different (Figure 8C). We also compared the immune suppressive potential of MDSCs in the recipients with GVHD or tumor relapse mice. At lower T cell-to-MDSC ratio, the MDSCs from the recipients with tumor relapse were more potent than those with GVHD (Supplementary Figure S5).

DISCUSSION

Previous work by others suggests that MDSCs accumulate in murine models of allogeneic BMT [17,34], but the relationships between MDSC accumulation, GVHD severity, and tumor relapse was not clear. Here, we systemically studied the accumulation of MDSCs in allogeneic and syngeneic BMT recipients. We found that MDSCs accumulated in both syngeneic and allogeneic BMT mice in the first 2 weeks, which indicates the initial MDSC accumulation was not related to GVHD but rather resulted from TBI-induced stress at an early stage of BMT. The proinflammatory cytokine release initiated by TBI, including IFN-γ, G-CSF, IL-1β, and IL-6, makes an ideal microenvironment for the accumulation and activation of MDSCs [35-37]. In the absence of GVHD, those elevated proinflammatory cytokines gradually returned to basal levels in parallel with the recovery of host tissue. Accordingly, MDSC percentage in mice declined to basal levels at week 4. Once GVHD developed, the escalated proinflammatory cytokines continued stimulating the expansion and accumulation of MDSCs (as seen in recipients transplanted with allogeneic BM plus T cells). GVHD also increased MDSC percentage by interfering with bone marrow and spleen reconstitution at later time points. These results indicate that GVHD development supports the constant accumulation of MDSCs over time. In fact, we observed a positive correlation between the levels of MDSCs in peripheral blood and the severity of GVHD.

In clinical hematopoietic cell transplantation, tumor relapse is a major threat to patients. We studied whether tumor relapse further increases MDSC accumulation in allogeneic BMT recipients. Our results showed that tumor relapse stimulated the additional accumulation of MDSCs in the peripheral blood and spleens of recipients after

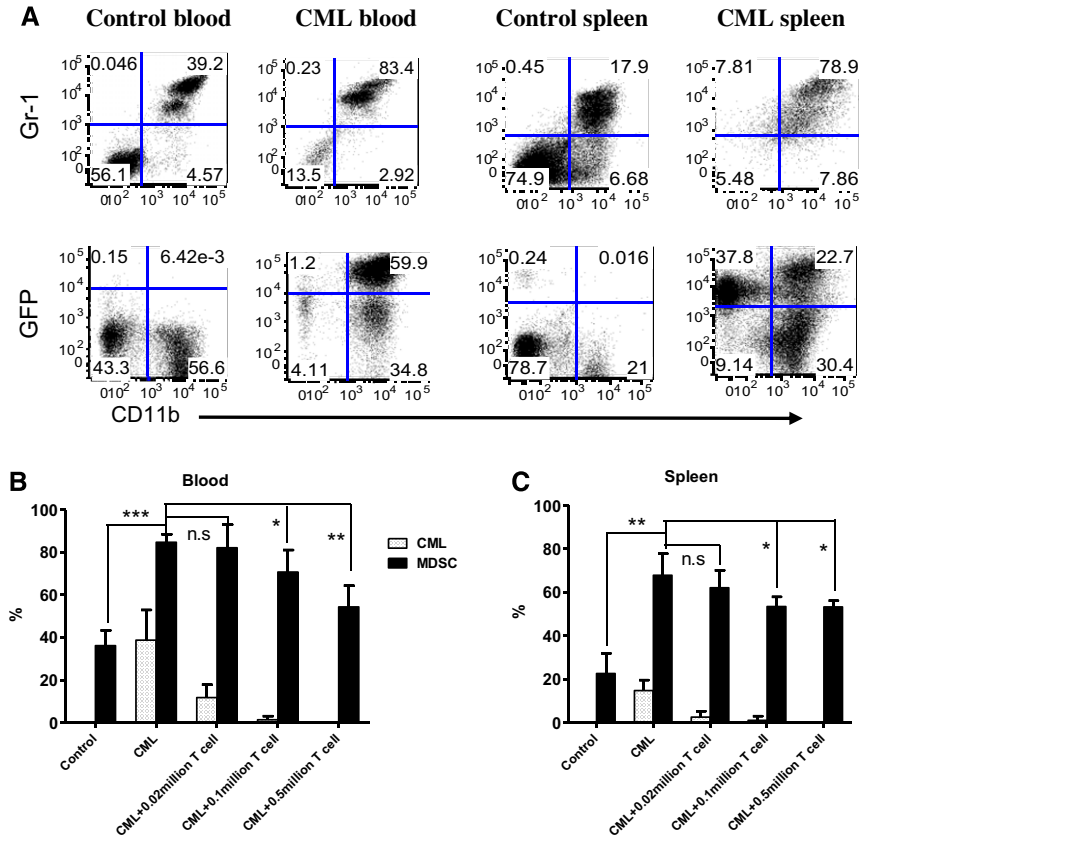

Figure 7. Spontaneous tumor (CML) relapse increased MDSC accumulation. BALB/c mice (n = 4) were lethally irradiated and transplanted with B6 TCD-BM alone as control or plus 2 $\times 10^6$ CML-BM and CML-splenocytes to mimic CML relapse in the recipient mice. MDSC and CML cells in blood and spleen were analyzed at week 3. (A) CML cell and MDSC in the blood and spleen of control mice and CML mice. (B) The percentage of MDSC in donor cells and the percentage of CML cells among total white blood cells. (C) The percentage of MDSC in donor cells and the percentage of CML cells in total spleen cells. One representative experiment from 3 independent experiments was shown. n.s. indicates no significant difference, * $P < .05$, ** $P < .01$, *** $P < .001$.

allogeneic BMT. Therefore, monitoring blood MDSCs may predict tumor relapse.

In this study, Gr-1 and CD11b were used to identify MDSCs, although other cell types may also express these 2 markers, such as neutrophils and granulocytes. Hence, phenotype alone was not enough to identify MDSCs, and the gold standard was the immune suppressive functional study. We found that MDSCs isolated from both allogeneic and syngeneic BMT recipients were indeed immune suppressive. The immune suppressive efficiencies were quite similar at week 2 but differed significantly at week 4 indicating the occurrence of GVHD not only kept MDSC accumulation constant but also maintained MDSC immune suppressive efficiency at high levels. Previous studies have attributed MDSCs' suppressive functions to arginase-1, inducible nitric oxide synthase, reactive oxygen species, and/or peroxynitrite [25-27,38]. Here we demonstrated that the suppressive functions of MDSCs in allogeneic BMT were arginase-1 and reactive oxygen species dependent. We did not see a reversal effect of L-NMMA on MDSCs. The possible reason is that we tested the combination of both granulocytic and monocytic MDSCs, whereas L-NMMA was only effective on monocytic MDSCs [23]. Considering the larger proportion (>75%) of granulocytic MDSCs in the mixture, it is possible that the effect through L-NMMA was not visible.

MDSCs accumulate in many pathological conditions and facilitate the evasion of tumor immunity in patients; however, studies have indicated that MDSCs may play a regulatory role in disease progression. In allogeneic BMT, exogenous MDSCs alleviate GVHD in mice [18,19]. Therefore, manipulating MDSCs may be a promising strategy to regulate GVHD after allogeneic BMT. Here we showed that removal of MDSC with ATRA significantly accelerated GVHD. We rationalize that ATRA treatment removed MDSCs by either depleting or differentiating MDSCs. Because acute GVHD is initiated by host APCs, and donor APCs contribute to the maximal GVHD [16], it is possible that increased conversion of donor APCs by ATRA treatment might also attribute to accelerated GVHD. Our data showed ATRA did not affect T cell proliferation under the stimulation of anti-CD3 and anti-CD28 Abs in vitro, which indicated T cells might not be the key target for ATRA treatment, although we cannot exclude this possibility.

Given that accumulated MDSCs in BMT recipients were sufficient to suppress allogeneic T cell proliferation in vitro, it is not clear why they were incapable of preventing GVHD in vivo. One possibility is that the accumulation of MDSCs occurred later than the activation and proliferation of allogeneic T cells in vivo, and thus those MDSCs had no chance to control ongoing GVHD. In support of this hypothesis, Paraiso et al. showed that SH2 domain–containing inositol 5-phosphatase (SHIP) deficiency resulted in a rapid and significant expansion of MDSCs in peripheral lymphoid tissues of recipient mice before receiving a T cell-replete BM graft, which led to the abrogation of acute GVHD [39]. This work and the work of others showed that MDSCs isolated in vivo from G-CSF–treated mice or MDSCs generated in vitro with various cytokines significantly prevented GVHD

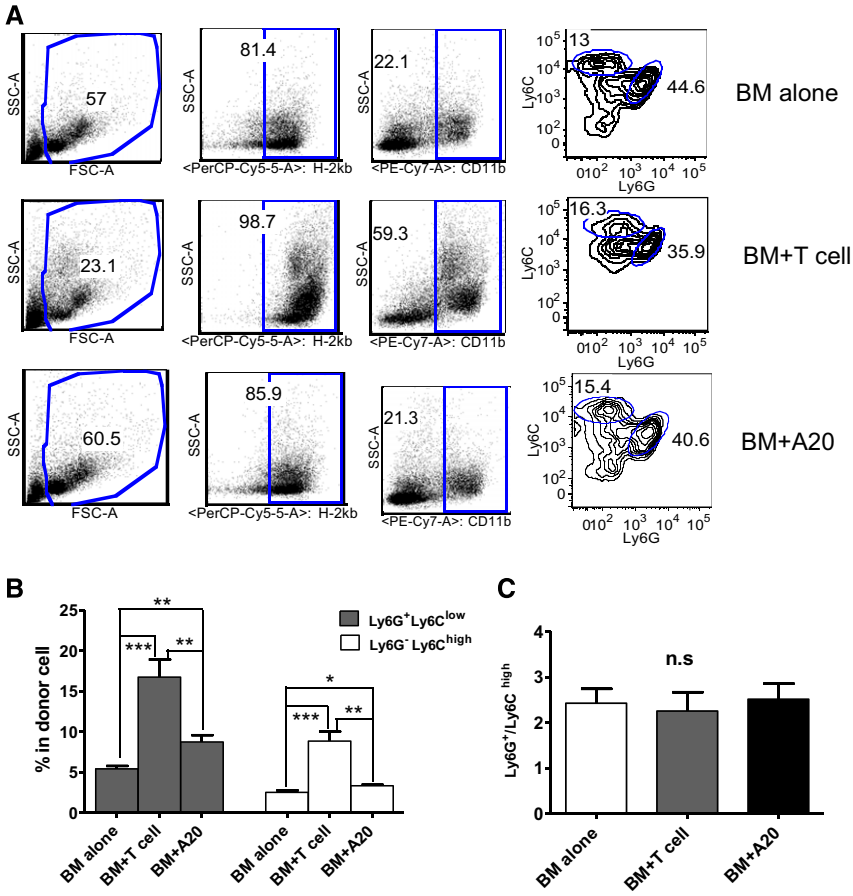

Figure 8. The composition of MDSC is similar in the spleen of GVHD or tumor-relapsed mice. BALB/c mice (n = 5) were lethally irradiated and underwent transplantation with B6 TCD-BM alone or plus 1×10^6 B6 T cells or 1×10^3 A20-Luc cells. Mice were euthanized on day 21, and the percentages of MDSC subpopulations in recipient spleens were analyzed. (A) Phenotype of donor-derived granulocytic ($CD11b^+Ly6G^+Ly6C^{low}$) and monocytic ($CD11b^+Ly6G^-Ly6C^{high}$) MDSCs. (B) Percentage of donor-derived granulocytic and monocytic MDSCs. (C) The ratio of granulocytic versus monocytic MDSC. n.s. indicates no significant difference, * $P < .05$, ** $P < .01$, *** $P < .001$.

[18,19]. Previous evidence indicates that delayed donor lymphocyte infusion causes less severe GVHD [40], which would support the idea that early accumulation of MDSCs before T cell infusion is required to suppress T cell activation and thus reduce GVHD development.

Importantly, Gr-1^+CD11b^+ cells isolated from PBS-treated mice BM accelerated GVHD, because the Gr-1^+CD11b^+ cells were physiologically immature myeloid cells, which further differentiated into DCs and macrophages posttransplantation and promoted GVHD development [30,41]. It is noteworthy that MDSC isolated from the spleen and bone marrow of G-CSF–treated mice showed a significantly different GVHD preventive effect (Figure 5D-F). The possible explanation is that there were more than 30% Gr-1^+CD11b^+ cells in bone marrow but less than 5% in spleen of normal mice. Because these cells are immature myeloid cells without suppressive function, a substantially higher proportion of these immature myeloid cells in bone marrow would dilute the functional MDSCs newly generated in mice treated with G-CSF, which resulted in a reduced efficacy of total MDSCs isolated from BM in the prevention of GVHD.

In summary, we showed that the accumulation of MDSCs was positively correlated with GVHD severity. Although these MDSCs had a regulatory activity to suppress T cell responses to alloantigens, they were not sufficient to control GVHD development under routine allogeneic BMT conditions. However, we provide evidence that adding functional MDSCs at the time of BMT significantly alleviated GVHD, whereas removal of MDSCs correlated with accelerated GVHD, indicting MDSCs have a direct relevance to GVHD development. It cannot be denied that MDSCs are a double-edged sword for allogeneic BMT patients, as they negatively regulate GVHD development but also facilitate tumor growth. Maintaining a delicate balance of MDSCs may present a challenging but promising approach for the control of GVHD and tumor relapse after allogeneic BMT.

ACKNOWLEDGMENTS

The authors thank Dr. Robert Negrin at Stanford for providing the luciferase transgenic mice, Dr. Warren Shlomchik at Yale for providing P210 bcr/abl vector, and Drs. Amer Beg and Esteban Celis for their critical comments on this work. The authors are grateful for the technical assistance provided by the Flow Cytometry and Mouse Core Facilities at the Moffitt Cancer Center.

Financial disclosure: Supported in part by Department of Defense pilot grant W81XWH-08-2-0101 and by National Institutes of Heath grants CA118116, CA143812, and AI 082685 to X.-Z.Y.

Conflict of interest statement: There are no conflicts of interest to report.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbmt.2013.01.008.

REFERENCES

1. Young MR, Newby M, Wepsic HT. Hematopoiesis and suppressor bone marrow cells in mice bearing large metastatic Lewis lung carcinoma tumors. *Cancer Res*. 1987;47:100-105.
2. Buessow SC, Paul RD, Lopez DM. Influence of mammary tumor progression on phenotype and function of spleen and in situ lymphocytes in mice. *J Natl Cancer Inst*. 1984;73:249-255.
3. Seung LP, Rowley DA, Dubey P, Schreiber H. Synergy between T cell immunity and inhibition of paracrine stimulation causes tumor rejection. *Proc Natl Acad Sci USA*. 1995;92:6254-6258.
4. Giordanengo L, Guiñazú N, Stempin C, et al. Cruzipain, a major *Trypanosoma cruzi* antigen, conditions the host immune response in favor of parasite. *Eur J Immunol*. 2002;32:1003-1011.
5. Goñi O, Alcaide P, Fresno M. Immunosuppression during acute *Trypanosoma cruzi* infection: involvement of Ly6G (Gr1(+))CD11b(+) immature myeloid suppressor cells. *Int Immunol*. 2002;14:1125-1134.
6. Zhu B, Bando Y, Xiao S, et al. CD11b+Ly-6C(hi) suppressive monocytes in experimental autoimmune encephalomyelitis. *J Immunol*. 2007;179: 5228-5237.
7. Holda JH, Maier T, Claman HN. Graft-vs-host reactions (GVHR) across minor murine histocompatibility barriers. I. Impairment of mitogen responses and suppressor phenomena. *J Immunol*. 1985;134: 1397-1402.
8. Holda JH, Maier T, Claman HN. Murine graft-versus-host disease across minor barriers: immunosuppressive aspects of natural suppressor cells. *Immunol Rev*. 1985;88:87-105.
9. Maier T, Holda JH, Claman HN. Graft-vs-host reactions (GVHR) across minor murine histocompatibility barriers. II. Development of natural suppressor cell activity. *J Immunol*. 1985;135:1644-1651.
10. Oseroff A, Okada S, Strober S. Natural suppressor (NS) cells found in the spleen of neonatal mice and adult mice given total lymphoid irradiation (TLI) express the null surface phenotype. *J Immunol*. 1984;132: 101-110.
11. Strober S. Natural suppressor (NS) cells, neonatal tolerance, and total lymphoid irradiation: exploring obscure relationships. *Annu Rev Immunol*. 1984;2:219-237.
12. Pak AS, Wright MA, Matthews JP, et al. Mechanisms of immune suppression in patients with head and neck cancer: presence of CD34(+) cells which suppress immune functions within cancers that secrete granulocyte-macrophage colony-stimulating factor. *Clin Cancer Res*. 1995;1:95-103.
13. Young MR, Kolesiak K, Wright MA, Gabrilovich DI. Chemoattraction of femoral CD34+ progenitor cells by tumor-derived vascular endothelial cell growth factor. *Clin Exp Metastasis*. 1999;17:881-888.
14. Koyama M, Kuns RD, Olver SD, et al. Recipient nonhematopoietic antigen-presenting cells are sufficient to induce lethal acute graft-versus-host disease. *Nat Med*. 2012;18:135-142.
15. Shlomchik WD, Couzens MS, Tang CB, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science*. 1999;285:412-415.
16. Matte CC, Liu J, Cormier J, et al. Donor APCs are required for maximal GVHD but not for GVL. *Nat Med*. 2004;10:987-992.
17. Billiau AD, Fevery S, Rutgeerts O, et al. Transient expansion of Mac1+Ly6-G+Ly6-C+ early myeloid cells with suppressor activity in spleens of murine radiation marrow chimeras: possible implications for the graft-versus-host and graft-versus-leukemia reactivity of donor lymphocyte infusions. *Blood*. 2003;102:740-748.
18. Highfill SL, Rodriguez PC, Zhou Q, et al. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. *Blood*. 2010;116:5738-5747.
19. Joo YD, Lee SM, Lee SW, et al. Granulocyte colony-stimulating factor-induced immature myeloid cells inhibit acute graft-versus-host disease lethality through an indoleamine dioxygenase-independent mechanism. *Immunology*. 2009;128:e632-e640.
20. Cooke KR, Kobzik L, Martin TR, et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation. I. The roles of minor H antigens and endotoxin. *Blood*. 1996;88: 3230-3239.
21. Matte CC, Cormier J, Anderson BE, et al. Graft-versus-leukemia in a retrovirally induced murine CML model: mechanisms of T cell killing. *Blood*. 2004;103:4353-4361.
22. Liang Y, Liu C, Djeu JY, et al. Beta2 integrins separate graft-versus-host disease and graft-versus-leukemia effects. *Blood*. 2008;111: 954-962.
23. Movahedi K, Guilliams M, Van den Bossche J, et al. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood*. 2008;111: 4233-4244.
24. Dietlin TA, Hofman FM, Lund BT, et al. Mycobacteria-induced Gr-1+ subsets from distinct myeloid lineages have opposite effects on T cell expansion. *J Leuk Biol*. 2007;81:1205-1212.
25. Rodríguez PC, Ochoa AC. Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. *Immunol Rev*. 2008;222:180-191.
26. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol*. 2005;5:641-654.
27. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI. Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. *J Immunol*. 2004;172: 989-999.
28. Nagaraj S, Gupta K, Pisarev V, et al. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med*. 2007;13: 828-835.
29. Lee JM, Seo JH, Kim YJ, et al. The restoration of myeloid-derived suppressor cells as functional antigen-presenting cells by NKT cell help and all-trans-retinoic acid treatment. *Int J Cancer*. 2012;131(3): 741-751.
30. Kusmartsev S, Cheng F, Yu B, et al. All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. *Cancer Res*. 2003;63:4441-4449.
31. Mirza N, Fishman M, Fricke I, et al. All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res*. 2006;66:9299-9307.
32. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. 2009;9:162-174.
33. Adeegbe D, Serafini P, Bronte V, et al. In vivo induction of myeloid suppressor cells and CD4(+)Foxp3(+) T regulatory cells prolongs skin allograft survival in mice. *Cell Transplant*. 2011;20: 941-954.
34. Luyckx A, Schouppe E, Rutgeerts O, et al. Subset characterization of myeloid-derived suppressor cells arising during induction of BM chimerism in mice. *Bone Marrow Transplant*. 2012;47(7):985-992.
35. Hill GR, Crawford JM, Cooke KR, et al. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. *Blood*. 1997;90:3204-3213.
36. Singh VK, Fatanmi OO, Singh PK, Whitnall MH. Role of radiation-induced granulocyte colony-stimulating factor in recovery from whole body gamma-irradiation. *Cytokine*. 2012;58:406-414.
37. Zhang M, Yin L, Zhang K, et al. Response patterns of cytokines/chemokines in two murine strains after irradiation. *Cytokine*. 2012;58: 169-177.
38. Vickers SM, MacMillan-Crow LA, Green M, et al. Association of increased immunostaining for inducible nitric oxide synthase and nitrotyrosine with fibroblast growth factor transformation in pancreatic cancer. *Arch Surg*. 1999;134:245-251.
39. Paraiso KH, Ghansah T, Costello A, et al. Induced SHIP deficiency expands myeloid regulatory cells and abrogates graft-versus-host disease. *J Immunol*. 2007;178:2893-2900.
40. Johnson BD, Becker EE, Truitt RL. Graft-vs.-host and graft-vs.-leukemia reactions after delayed infusions of donor T-subsets. *Biol Blood Marrow Transplant*. 1999;5:123-132.
41. Nefedova Y, Fishman M, Sherman S, et al. Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells. *Cancer Res*. 2007;67:11021-11028.